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## Review

# Integrins during evolution: Evolutionary trees and model organisms

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## ABSTRACT

The integrins form a large family of cell adhesion receptors. All multicellular animals express integrins, indicating that the family evolved relatively early in the history of metazoans, and homologous sequences of the component domains of integrin  $\alpha$  and  $\beta$  subunits are seen in prokaryotes. Some integrins, however, seem to be much younger. For example, the  $\alpha$ 1 domain containing integrins, including collagen receptors and leukocyte integrins, have been found in chordates only. Here, we will discuss what conclusions can be drawn about integrin function by studying the evolutionary conservation of integrins. We will also look at how studying integrins in organisms such as the fruit fly and mouse has helped our understanding of integrin evolution–function relationships. As an illustration of this, we will summarize the current understanding of integrin involvement in skeletal muscle formation.

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## 1. Introduction

### 1.1. Cell adhesion during evolution and development

Cell adhesive mechanisms act during development to regulate tissue formation, and multicellular organisms have developed increasingly refined mechanisms for regulating cell adhesive events in the course of their evolution. Interactions with the extracellular matrix (ECM) via cell surface receptors have been shown to be

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important during several steps in embryonic development. Cell–cell contacts need to be formed and broken in a dynamic manner during the early steps in organogenesis, and cell migration events are needed to direct cells to the right places during remodelling and growth phases. Towards the end of organogenesis, a complete set of static interactions are needed to keep the cells in place in the newly formed tissues. Most importantly, another set of contacts is then required to allow for postnatal growth, being finally replaced by stable contacts that keep the cells in place in the adult tissues.

The demands on adhesive events of the type that need to be regulated also increase with the formation of new physiological systems, such as a closed circulatory system or a complete immune system [1,2], in both of which cells circulating in the body constantly need to be ready to engage in transient interactions such as in immune surveillance, blood clotting and inflammation. The need to change and adapt cell adhesive interactions must also be retained in readiness for regeneration events following tissue damage, often involving the recruitment of stem cells of various types.

## 1.2. Integrins

Integrins have many roles during development. Since all nucleated cells in the body have a specific repertoire of integrins, these roles are complex and are integrated with the specific functions of the cells in different tissues.

The functions of integrins at the cellular level can be grouped into those related to their roles as mechanical links in cell adhesion and migration, and their signalling function [3]. The integrin-dependent effect on total signalling in a certain cell type can influence cytoskeletal arrangements, transcriptional activity and a variety of metabolic reactions. These roles then become even more complex in a tissue context, as illustrated by findings that integrins can regulate the activity of paracrine loops of secreted growth factors [4–6].

When trying to understand the role of integrins in developmental processes, two types of insight can be gained from studying model organisms located at different positions in an evolutionary tree: a detailed understanding of a particular integrin orthologue in another species, or general knowledge about how integrins influence a developmental process, knowledge which is not necessarily tied to a specific integrin.

Examples of the latter include the general importance of integrins for skeletal muscle integrity in *Drosophila* and mammals. Recent fruit fly data show that integrins are important for setting up the stem cell niche [7,8], and it is an exciting possibility that the findings made regarding the *Drosophila* testis might also have implications for future stem cell-based therapeutic approaches in humans.

In this review we will discuss what can be learned about integrin structure and function from comparisons of integrins between species. We will illustrate the usefulness of studying different model organisms in order to understand integrin function in a specific tissue by comparing skeletal muscle development in the fruit fly (*Drosophila melanogaster*), and mouse (*Mus musculus*). Several excellent reviews on the role of integrins in *Caenorhabditis elegans* exist [3,9], but in the current review we have chosen not to discuss this nematode. Some additional animal models will be mentioned in relation to their potential as model organisms for future use.

## 2. Integrins during evolution

### 2.1. General

Integrins are heterodimers composed of one  $\alpha$  and one  $\beta$  subunit. Present-day mammals are known to express 18 different integrin  $\alpha$  subunits and 8  $\beta$  subunits [10]. The model of integrin synthesis indicates that the promiscuous subunits, such as  $\beta 1$  or  $\alpha V$ , are produced in large excess when compared to the other subunits [11,12].

In the endoplasmic reticulum integrin subunits find their binding partners and form heterodimers. However, only 24 different  $\alpha\beta$  combinations are observed in human (Table 1) and the monomeric integrins never reach the cell surface. The integrin  $\alpha$  and  $\beta$  subunits have separate evolutionary histories, but the evolution of new heterodimers may have required concomitant changes in both the  $\alpha$  and  $\beta$  subunits.

The  $\alpha$  and  $\beta$  subunits in human have clearly recognizable homologues in organisms that diverged from the tree of life long before the appearance of the first vertebrates. Indeed, the origins of the subunits or component pieces appear early in evolution and predate the chordate line and in the case of von Willebrand factor A (vWFA) they are found in prokaryotes [13,14]. We have also seen that large amino-terminal regions of both the  $\alpha$  and  $\beta$  subunits that respectively code for the  $\beta$  propeller and the  $\beta I$  domain in the integrins are found in protein sequences from bacteria (see below).

The general function of the integrins – a role in cell adhesion and communication – appears to have been established early as well and

**Table 1**  
Mammalian integrins.

Integrin	Ligands	Location of defects in knock-outs and/or main expression sites
<i>Human integrins containing a PS1 group <math>\alpha</math> subunit</i>		
$\alpha 3\beta 1$	Laminins (collagens)	Skin, kidney, lung, cortex
$\alpha 6\beta 1$	Laminins, ADAMs	Gametes, macrophages, platelets
$\alpha 6\beta 4$	Laminins	Skin (hemidesmosomes)
$\alpha 7\beta 1$	Laminins	Muscle
<i>Human integrins containing a PS2 group <math>\alpha</math> subunit</i>		
$\alpha 5\beta 1$	Fibronectin (RGD)	Embryonic development (blood vessels)
$\alpha 8\beta 1$	Fibronectin, vitronectin, tenascin C, osteopontin, nefronectin (RGD)	Kidney, inner ear
$\alpha V\beta 1$	Fibronectin, vitronectin (RGD)	Not clear, whether expressed <i>in vivo</i>
$\alpha V\beta 3$	Fibrinogen, fibronectin, vitronectin, tenascin C, osteopontin, bone sialoprotein (RGD), MMP-2	Bone (osteoclasts)
$\alpha V\beta 5$	Vitronectin (RGD)	Eye (retinal phagocytosis), bone (osteoclastogenesis)
$\alpha V\beta 6$	Fibronectin, TGF- $\beta$ -LAP (RGD)	Skin, lung (collagen accumulation)
$\alpha V\beta 8$	Vitronectin (RGD)	Vascular development
$\alpha IIb\beta 3$	Fibrinogen (RGD, GAKQAGDV), Fibronectin, vitronectin (RGD)	Platelets
<i>Human integrins containing an <math>\alpha 4/\alpha 9</math> group <math>\alpha</math> subunit</i>		
$\alpha 4\beta 1$	Fibronectin, VCAM	Embryonic development (heart)
$\alpha 4\beta 7$	Fibronectin, VCAM, MadCaM	Peyer's patch (immune system)
$\alpha 9\beta 1$	Tenascin C, osteopontin, ADAMs, factor XIII, VCAM, VEGF-C, VEGF-D	Lymphangiogenesis
<i>Human integrins containing an <math>\alpha</math> subunit with an <math>\alpha I</math> domain</i>		
Collagen receptor subgroup		
$\alpha 1\beta 1$	Collagens, semaphorin 7A, (laminins)	Mesenchymal tissues
$\alpha 2\beta 1$	Collagens, tenascin C, (laminins)	Platelets, epithelium, mast-cells (mesenchymal tissues)
$\alpha 10\beta 1$	Collagens	Cartilage
$\alpha 11\beta 1$	Collagens	Periodontal ligament
Leukocyte integrin subgroup		
$\alpha D\beta 2$	ICAM, VCAM	Eosinophils
$\alpha M\beta 2$	ICAM, VCAM, iC3b, factor X, fibrinogen	Leukocytes (phagocytosis)
$\alpha L\beta 2$	ICAM	Leukocytes (recruitment)
$\alpha X\beta 2$	Fibrinogen, plasminogen, heparin, iC3b	Leukocytes
$\alpha E\beta 7$	E-cadherin	Skin, Gut (immune system)

The heterodimers have been organized based on the phylogenetic subgroup of the  $\alpha$  subunit.

has been well characterized in several invertebrates (for a review, see [15]) such as in *C. elegans* where two  $\alpha$  subunits (Ina-1, Pat-2) and one  $\beta$  subunit (Pat-3) [16–18] are found and five  $\alpha$  and two  $\beta$  subunits in *Drosophila* [19–25].

In vertebrates, the integrin subunits have diversified to accommodate additional functions with roles that likely relate to the large changes that occurred during chordate evolution in the transition to cartilaginous and bony internal structures, a dual innate and adaptive immune system and the high-pressure circulatory system that characterize the vertebrate lineage. Thus, the vertebrate integrin subunits did not appear suddenly, but arose from homologous sequences and domains that preexisted their functions in cell adhesion in the first metazoans and existing within the protists and prokaryotes.

## 2.2. Integrin $\alpha$ and $\beta$ subunits

The mammalian  $\alpha$  subunits can be divided into four different main groups but do not have representatives within a fifth cluster comprising sequences from insects (Fig. 1) [13,26–29]. One subfamily of integrin  $\alpha$  subunits ( $\alpha 3$ ,  $\alpha 7$ ,  $\alpha 6$ ) is evolutionarily related to *Drosophila* PS1 proteins (Table 1). Sequences from invertebrates, including *C. elegans* (Ina-1), *Drosophila* (PS1), and the urochordates *Polyandrocarpa misakiensis* and *Ciona intestinalis* (tunicate; ascidian; sea squirt), cluster as outliers within this grouping indicating that they are ancestral to but not direct functional orthologues of the present-day mammalian subunits. *Drosophila* PS1 and the mammalian orthologues have all evolved to recognize members of the laminin family [30]. Laminins are important structural proteins in basement membranes. Mammalian PS1 group  $\alpha$  subunits can form heterodimers with  $\beta 1$  or in one case with  $\beta 4$  subunits. These integrins are important for the integrity of tissues, including kidney, skin and muscle.

Another subgroup of integrin  $\alpha$  subunits, namely  $\alpha 1b$ ,  $\alpha V$ ,  $\alpha 5$ , and  $\alpha 8$ , is structurally related to the *Drosophila* PS2 protein (Table 1). As with the PS1 cluster, the known invertebrate sequences cluster as

ancestral homologues but not direct orthologues of the mammalian sequences (asterisks in Fig. 1 indicate the relative branch points for invertebrate sequences on the generalized tree for the vertebrate integrin  $\alpha$  subunits). Other invertebrate sequences include those of *C. elegans* (Pat-2), the echinoderms *Stroglyocentrotus purpuratus* and *Lytechinus variegatus* (sea urchins), and the urochordates *Halocynthia roretzi* and *C. intestinalis*. The  $\alpha$  integrin from a very early metazoan, the sponge *Geodia cydonium* [31], is also reported to cluster near the PS2 subgroup [27].

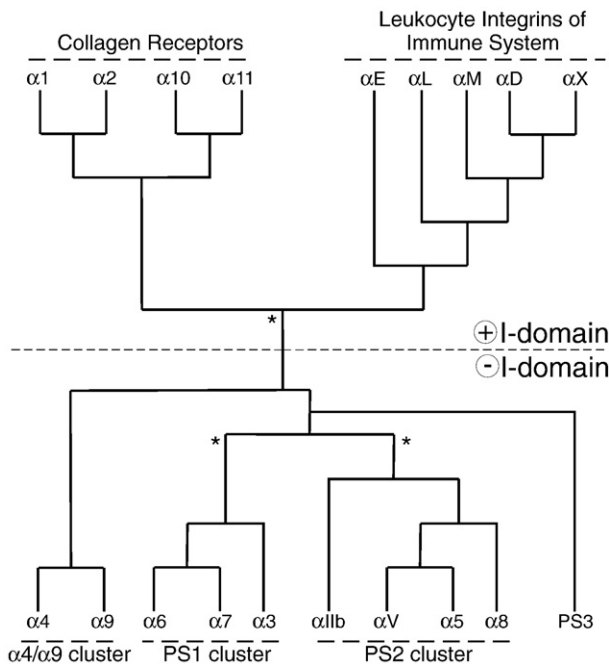
The receptors of the PS2 group have evolved to recognize a short motif, such as the classic RGD sequence, in extracellular matrix (ECM) proteins. In mammals, there are several RGD-containing ECM proteins, such as fibronectin. *Drosophila* has no orthologue for fibronectin, but PS2 recognizes other ECM proteins, such as tigrin [32]. In mouse, one of the RGD-dependent mammalian integrins, that is  $\alpha 5$ , seems to be indispensable for early embryonic development [33], stressing the fundamental nature of RGD-dependent cell adhesion and locomotion. *G. cydonium* contains a putative multiadhesive protein possibly containing a fibronectin FN<sub>3</sub> module, a sequence (KILDA) similar to EILDV that is recognized by some mammalian integrins, but no RGD motif [34].

Other mammalian integrins in the PS2 group have much more specific functions in bone cells, kidney cells, vascular development etc. (Table 1). Interestingly, in vertebrates the RGD motif is also found in non-ECM proteins. Cell adhesion to plasma fibrinogen is integrin-mediated and RGD-dependent. In addition to the RGD motif  $\alpha IIb\beta 3$  integrin on platelets can recognize another similar sequence in fibrinogen, namely GAKQADV [35]. Furthermore, the activation of a growth factor, namely the latent form of transforming growth factor  $\beta$  (TGF- $\beta$ ), may be mediated by integrins that recognize a RGD motif in the LAP (latency associated peptide) domain of latent TGF- $\beta$  [36]. These examples suggest that integrins have also received new functions due to evolution in other protein families.

While the PS1 and PS2 group integrins have been found in many invertebrates, including *C. elegans*, a third subgroup of *Drosophila* integrin  $\alpha$  subunits, named PS3, with representatives also seen in the butterfly *Pseudoplusia includens* [28], seems to be specific for insects. Two mammalian  $\alpha$  subunits, namely  $\alpha 4$  and  $\alpha 9$  can be considered as a separate subgroup (Table 1). These integrins recognize, in addition to ECM proteins, certain plasma proteins, counter receptors belonging to the immunoglobulin superfamily and vascular endothelial cell growth factors [37].

A structurally and functionally unique subgroup of integrin  $\alpha$  subunits is formed by integrins that contain a specific “inserted” or “ $\alpha I$ ” domain. Because of the structural similarity to the von Willibrand factor “A” domain these domains are also often called integrin “ $\alpha A$ ” domains.  $\alpha I$  domains form the ligand recognition part of the corresponding integrins. In mammals, nine out of 18  $\alpha$  subunits have the  $\alpha I$  domain. Four  $\alpha I$  domain integrins are collagen receptors (reviewed in: [38,39]), while the other five are expressed on leukocytes (reviewed in [40]) and they recognize counter receptors, such as E-cadherins or intercellular and vascular adhesion molecules (ICAMs or VCAMs, correspondingly). Some of them also bind to plasma proteins such as fibrinogen or component iC3b in the complement system (Table 1). Similarly the collagen receptor subgroup of integrins have at least a limited ability to bind to other ECM proteins including the laminins. Semaphorin 7A participates in the regulation of axon guidance and activation of inflammatory cells and was recently described to be recognized by the  $\alpha 1\beta 1$  collagen receptor [41].

Interestingly, the  $\alpha I$  domain integrins have not been found in invertebrates, such as insects and worms. Recently, up to eight  $\alpha I$  domain integrin genes were recognized in the genome of *C. intestinalis*, representing the most primitive chordates [26,28,29,42]. The *Ciona*  $\alpha I$  domain integrins form a separate group of  $\alpha I$  domain integrins that is different when compared to either mammalian



**Fig. 1.** Generalized tree for the integrin  $\alpha$  subunits present in human that are most probably found within the entire vertebrate lineage. The PS3 cluster only contains invertebrate sequences but some invertebrate sequences also cluster as outliers of the vertebrate clusters (asterisks indicate the branch point). The earliest diverging  $\alpha$  subunits containing an I domain appear in the tunicates as outliers to both the collagen receptor and leukocyte clusters. Branch lengths are arbitrary.

collagen receptors or leukocyte integrins [26,28]. The functional testing of *Ciona* integrin  $\alpha$  domains has indicated that they cannot recognize triple helical, GFOGER (O=hydroxyproline) sequence containing peptides [42] in contrast to all vertebrate collagen receptor  $\alpha$  domains that can [43,44]. At the moment it is unclear what the actual function of these integrins is. However, the single  $\alpha$  domain integrin found in another ascidian species, *H. roretzi*, is reported to act as a complement receptor and participate in phagocytosis by hemocytes [45,46], but the sequence does not cluster as a direct orthologue of the mammalian type integrin I domains [13].

A sequence-based search revealed that an amino-terminal region from integrin  $\alpha$  subunits corresponding to the  $\beta$  propeller domain is recognizable in several bacterial sequences (unpublished). For example in a sequence from *Nitrosococcus oceanii* (accession code YP344083; [47]). This similarity is probably why the sequence was automatically labeled as “Integrin alpha chain” even though the sequence similarities appear to be limited to the  $\beta$  propeller region of integrin chains.

The integrin  $\beta$  subunits seem to form three major phylogenetic branches (Fig. 2), with those present in vertebrates termed groups A ( $\beta$ 1,  $\beta$ 2 and  $\beta$ 7) and B ( $\beta$ 3– $\beta$ 6 and  $\beta$ 8) and a third branch containing only invertebrate sequences [28]; this segregation agrees well with the earlier phylogenetic classification by Hughes [27] and the branching orders that have been reported [13,26–29]. Ewan et al. [26] have considered the  $\beta$ 4 branch to be a separate group of one, thus segregating the  $\beta$  subunits into Beta 1–3 plus a Beta ascidian group. In our three-branch classification, branch A contains  $\beta$  subunits that in human are associated with most of the alpha subunits and from the PS1, PS2 and I domain clusters: promiscuous  $\beta$ 1 associates with  $\alpha$ 1– $\alpha$ 11 and  $\alpha$ V;  $\beta$ 2 with  $\alpha$ D,  $\alpha$ L,  $\alpha$ M, and  $\alpha$ X; and  $\beta$ 7 with  $\alpha$ 4 and  $\alpha$ E. Branch B contains the  $\beta$ 3,  $\beta$ 5,  $\beta$ 6 and  $\beta$ 8 subunits that can each form a heterodimer with  $\alpha$ V (PS2),  $\beta$ 3 also forms heterodimer with  $\alpha$ IIb (PS2), whereas  $\beta$ 4 from branch B forms a heterodimer with  $\alpha$ 6 (PS1). A *Ciona*  $\beta$  subunit clusters with the  $\beta$ 4 integrins and as outliers to the cluster containing the  $\beta$ 1,  $\beta$ 2 and  $\beta$ 7 subunits (asterisks in Fig. 2), whereas multiple  $\beta$  subunits from *Ciona* and *Halocynthia* are part of the invertebrate group [26,28,29]. In addition to the  $\alpha$  integrin from the sponge *G. cydonium* [31], a  $\beta$  subunit from this sponge has also been sequenced [48], as well as  $\beta$  subunits from other Porifera including the coral *Acropora millepora* and sponge *O. tenuis*. Further-

more, sequences homologous to domains from integrin  $\beta$  subunits extend into the prokaryotes. For example, a sequence from the cyanobacterium *Trichodesmium erythraeum* has been automatically classified as a “ $\beta$ -like integrin” (accession code YP 721619). Our own alignment of the sponge and cyanobacterium sequences with the mammalian sequences clearly shows that the cyanobacterium sequence is homologous with a large portion of the first ~450 amino acids, which would correspond to the amino-terminal  $\beta$ A-domain and part of the hybrid domain of the vertebrate integrin  $\beta$  subunits. However, the EGF repeats apparently are not present in the *T. erythraeum* sequence as evidenced by the lack of repetitive conserved cysteine residues (unpublished).

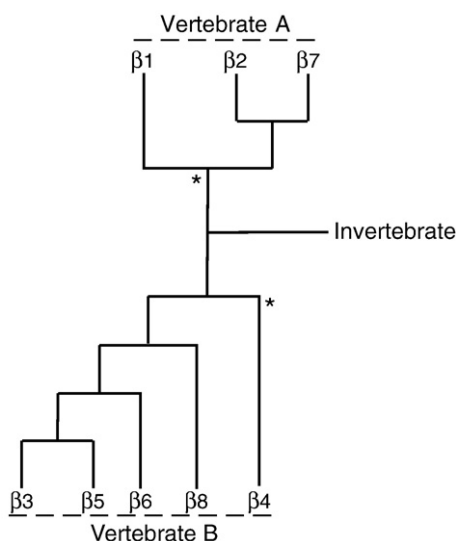
Three-dimensional structures of the ectodomain of human  $\alpha$ V $\beta$ 3 [49] and  $\alpha$ IIb $\beta$ 3 [50] have defined the structure of the individual domains and suggested that the integrin heterodimer undergoes large conformational changes related to outside-in and inside-out signaling events [49,51,52]. The sequence similarities and conservation of these domains in other species suggest that the overall structures have remained the same over evolutionary time and that the dynamic nature of the mammalian integrins may have been established already in the earliest recognizable integrins of the prokaryotes and the earliest metazoans. The structure of  $\alpha$ V $\beta$ 3 in complex with an RGD peptide [53] has pinpointed the binding site for this motif present on many proteins of the ECM; transmission electron microscopy has shown that this bent conformation of the ectodomain can also bind a fibronectin FN<sub>3</sub> fragment containing multiple domains [54]. No X-ray structure has yet been obtained for an  $\alpha$ I domain-containing integrin although quite reasonable models have been estimated based on the known X-ray structures of an I domain and the  $\alpha$ V $\beta$ 3 ectodomain [55]. Nishida et al. [56] have used electron microscopy to visualize the location of the I domain relative to the rest of the headpiece of both the  $\alpha$ X $\beta$ 2 and  $\alpha$ L $\beta$ 2 integrins in various conformations from bent to extended.

### 2.3. Origin of the vertebrate integrins

The available sequence data demonstrate that the integrins have a very early history. Both  $\alpha$  and  $\beta$  subunits have been found throughout the invertebrates, including one of the earliest metazoans, a sponge *G. cydonium*. Not only does the presence of integrin subunits appear to extend right through the invertebrates to the earliest metazoans, but large portions of the sequences from the  $\alpha$  and  $\beta$  subunits are detectable in prokaryotic sequences. Domains of the vWFA family have been used for a wide variety of functions throughout evolutionary history [13,14,57], not only in multicellular organisms but in unicellular eukaryotes and prokaryotes, too. Surprisingly, I-domain containing  $\alpha$  integrins are a much more recent phenomena (see below).

Although the picture is far from complete and limited by the genomes that have been sequenced, assembled and annotated, we do know that orthologues of the mammalian integrin subunits are present in the bony fish, where duplicate forms of both subunits have been observed (e.g., multiple  $\beta$ 1 orthologues in zebrafish *Danio rerio*, and duplicates of  $\alpha$ 5,  $\alpha$ 6,  $\alpha$ 10 and  $\alpha$ 11 in the pufferfish, *Takifugu rubripes*) [28,58], but orthologues of mammalian integrin subunits have not been detected in the tunicates whose genomes have been sequenced (e.g. *Ciona*). Furthermore, orthologues have not been reported for any of the organisms whose divergence precedes the chordate evolutionary line, whereas ancestral sequences are observed in tunicates as well as in other invertebrates that are clearly homologues of the  $\alpha$  and  $\beta$  integrins. As a result, we can bracket the origin of the present day vertebrate set as having diverged from the early integrin forms in species coming after the divergence of the tunicates and certainly before the divergence of the bony fish.

There are several aspects that make it difficult to pinpoint the moment in the history of life when vertebrate integrin forms were



**Fig. 2.** Generalized tree for the integrin  $\beta$  subunits present in human that are most probably found within the entire vertebrate lineage. Invertebrate sequence primarily cluster as a single group but tunicate sequences are seen to cluster as an outlier of the A group as a whole and as an outlier of the (4 integrins of the B group of vertebrate sequences. Branch lengths are arbitrary.



first established. Firstly, sequence data is currently lacking for species that diverged after the tunicates and before the bony fish. Secondly, several mass extinctions [59] especially during the Paleozoic era may have reduced the number of present data representatives quite considerably. Extant forms include amphioxus (a lancelet; Cephalochordata); the jawless vertebrates that include various species of lamprey and hagfish (Agnatha); and the Chondrichthyes (Elasmobranchii – sharks and rays, and the Holocephali – the chimaeras, (ratfish)). Nonetheless, the extant group of chordates representing the earliest divergence along the chordate line and containing authentic orthologues of the mammalian integrins should be established in the near future. Currently, the genomes of several species within Chondrichthyes are being sequenced: *Callorhinchus milii* (elephant shark or ghost shark), *Ginglymostoma cirratum* (nurse shark), *Squalus acanthias* (spiny dogfish) and the *Raja erinacea* (little skate). Given the large number of vertebrate features shared by sharks and rays in comparison with bony fish and the tetrapods, it would be surprising if orthologues of the human-type  $\alpha$  and  $\beta$  subunits were not eventually reported. Several genome-sequencing projects are also in progress on some intervening species between the Urochordates and Chondrichthyes: i.e., the cephalochordate *Branchiostoma floridae*, the Florida lancelet, and *Petromyzon marinus*, the sea lamprey.

The absence of any report of an  $\alpha$ L domain containing integrin in non-chordate invertebrates suggests that these integrins have clearly evolved quite late in the history of the metazoans. Based on their functional testing it can be speculated they have been recruited during the development of the immune system. Mammalian leukocyte integrins are essential for the immunity and some members in the collagen receptor subgroup of the  $\alpha$ L domain integrins also mediate leukocyte adhesion to collagens [38]. Furthermore, the mammalian  $\alpha$ 2 subunit has been linked to innate immunity [60].

The collagens themselves form a very large family of ECM proteins, which has been considered to play an essential role in the evolution of metazoans since their earliest origins [61–64]. Furthermore, collagens are among the most abundant proteins in tissues. Therefore, it is quite intriguing that the integrin-mediated mechanisms of direct collagen binding have not evolved earlier. The late appearance of these receptors may also be seen in the fact that the lack of collagen receptor integrins has very little effect on mouse development [38,39]. In addition to immunological functions, the collagen receptors seem to participate in the homeostasis of connective tissues [38,39]. The three-dimensional structures of vWFA domains [65–67] and  $\alpha$ L domains ( $\alpha$ L [68],  $\alpha$ M [69],  $\alpha$ 1 [70],  $\alpha$ 2 [71]) solved using X-ray crystallography have revealed their close structural similarity and location of the ligand binding metal ion binding MIDAS site. The  $\alpha$  subunit ligands bind to MIDAS of this domain, and the domain itself has been inserted within a repeat of the amino-terminal  $\beta$ -propeller domain. The complex of  $\alpha$ 2I with a collagen-like triple helix has defined the structural changes that occur in the collagen binding integrins, where a local conformational change involving helix C and shifting the domain into the “open” form is key to ligand recognition [55]. The presence of this helix defines the integrins of the collagen-binding subset:  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 10 and  $\alpha$ 11.

#### 2.4. What has driven the evolution of the integrins?

There are obvious large-scale developments that were essential to the development, survival and success of multicellular organisms: cell–cell interactions, cellular communication and development of large-scale supportive structures (i.e., cartilage and bone in the vertebrates) and the necessity to remodel a multicellular organism during the various stages of the life cycle; the development of the early innate immune response and rise of the adaptive immune response; and the development of a low-pressure circulatory system that is later replaced by the high-pressure circulatory system. Proteins of the ECM are key to many of the interactions that take place and

various proteins of the ECM have been established with the earliest metazoans.

The integrins have an early history that even predates their function in metazoan cell–cell communication. This is not unusual, since genes do not appear *de novo* when a new function at the protein level is taken on, but instead arise from preexisting genes via gene duplication, mutation, and combination and fusion of functional domains. In the case of the integrins, large changes in the organization of cells in multicellular organisms have gone hand-in-hand with changes in integrin subunit diversity, and a large increase of integrin subunits types appear in the vertebrates. Although some collagens have been present very early in metazoan evolution, recognition of collagens by integrins has appeared only relatively recently in one subset of the  $\alpha$  subunits that contain I domains. In contrast, the cell-adhesion system via the RGD motif appears to have a very early origin, possibly functioning in cell-substratum attachment in the unicellular protozoan *Neoparamoeba aestuarina* [72]. We have seen that representative sequences of both  $\alpha$  and  $\beta$  integrin subunits are found in bacteria but that their functions in these single-cell organisms are unknown at this time. With regard to the identification of the earliest appearance of true orthologues of the vertebrate subunits represented in extant chordates, the current genome sequencing projects should clarify this question within the very near future.

### 3. Studies of integrin function in the skeletal muscle of model organisms

#### 3.1. Introduction

Cell adhesion is a fundamental process during early embryonic events such as gastrulation, somitogenesis and myogenesis. For each of these morphogenetic processes cell adhesion must be closely regulated. With chordates and vertebrates new organ systems are introduced and most protein families diversify to become subspecialized as discussed in Section 2. In higher vertebrates, identifying the function of a large gene family in a particular tissue can be difficult, and here model organisms with smaller genomes such as *Drosophila* offer simpler alternatives to study principles of molecular mechanisms. Whereas many aspects of myogenesis have changed during evolution, some fundamental cell adhesive events have remained surprisingly intact. As some cells in vertebrate muscle tissues needed to interact with fibrillar collagens, the integrins on these cells have evolved to include new domains that enable these interactions. Muscle offers a fascinating tale of how molecular principles have evolved in tissues and have been conserved, but also of how protein families have subspecialized in order to engage in new functions. Below we will summarize some current state-of-the art knowledge pertaining to muscle cell adhesion, focusing on fruit fly and mouse.

#### 3.2. *D. melanogaster*

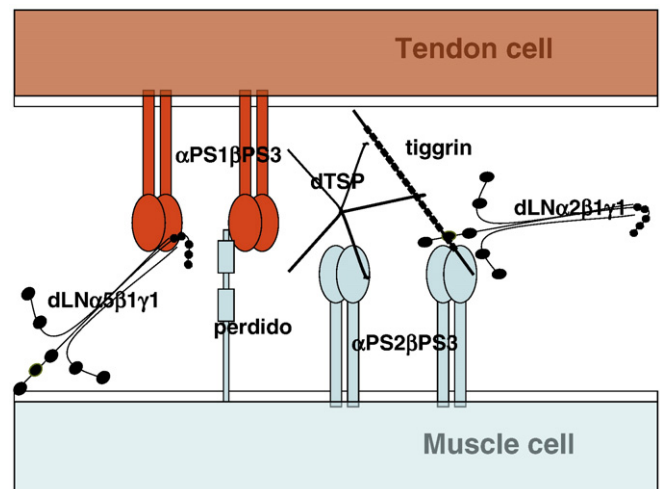
The fruit fly goes through a number of developmental stages such as egg, larva and pupa before the emergence of a mature insect. The relative ease with which one can obtain mutants and the availability of tools for analysing genetic interactions have made *Drosophila* a favourite model organism among biologists. Another major advantage is the smaller size of the genome, with correspondingly smaller gene families, thus causing less redundancy problems when analysing gene function. Studies of integrin function in *Drosophila* have focused on the formation of the midgut, muscle and wing [9,73], but some elegant work on axon guidance [74,75] and germ stem cells has also been performed more recently [7,8]. Recent data on the role of integrins during the establishment of a stem cell niche in the *Drosophila* testis [7,8] illustrate why clinicians involved with stem cell therapy should keep an eye on the fly literature. These data indicate that integrins play an important role in the correct positioning of the stem cell niche

within the tissue. If this is a conserved biological principle, it might have consequences for stem cell therapy and the use of artificial carrier materials. Depending on the source of the stem cells, one can thus envisage how stem cells might in the future have to be equipped with certain integrins in order to position themselves at the right place within the tissue.

As mentioned in Section 2 the integrin family in *Drosophila* is composed of 7 subunits, 5  $\alpha$  and two  $\beta$  subunits [76]. Some information on the integrin chains, which we will discuss in the context of muscle formation, i.e.  $\alpha$ PS1,  $\alpha$ PS2 and  $\beta$ PS3, is shown in Table 2.

In *Drosophila*, the formation of the embryonic somatic body muscles, which are later used mainly by the larva for crawling, involves MyoD-positive founder cells that prefigure the final muscle pattern and fusion of these with a second population of fusion-competent myoblasts [77–79]. The muscle cells then go through various stages in which they form contacts with specialized cells called tendon cells, which are connected to the cuticular exoskeleton. During this process the muscle cells extend and send out filipodial sensing extensions to make contact with the tendon cells [80]. In the absence of  $\beta$ PS integrins, cell fusion is defective, the rudimentary muscles show defective cytoskeletal attachments and the muscle cells come lose from their attachment points midway through embryogenesis [21,81–83]. Severe defects were also observed when integrin-deficient myoblasts were isolated and cultured *in vitro* [84].

Some molecular details of muscle attachment have remained obscure until recently. The muscle cells on the muscle side of the junction express the  $\alpha$ PS2 integrin chain, whereas the  $\alpha$ PS1 integrin subunit is expressed on the adjoining tendon cell side [85], and there is a basement membrane containing the integrin ligands tigrin [32], laminin  $\alpha 5\beta 1\gamma 1$  [86] and laminin  $\alpha 2\beta 1\gamma 1$  [87] in between the cell layers. *Drosophila* laminin with the composition  $\alpha 5\beta 1\gamma 1$  was the first  $\alpha$ PS1 $\beta$ PS ligand to be identified [30], while the RGD-containing proteins tigrin [32] and laminin with the composition  $\alpha 2\beta 1\gamma 1$  were identified as  $\alpha$ PS2 $\beta$ PS ligands in later *in vitro* studies [88]. Surprisingly, the incorporation of tigrin into the muscle matrix *in vivo* is largely normal in *mys* mutants [32], and absence of the laminin  $\alpha 5$  chain likewise has only relatively minor effects on somatic muscle attachments. On the other hand, the laminin  $\alpha 2$  chain appears to be crucial for proper muscle attachments to form [88,89]. Thus, although tigrin and laminin  $\alpha 5\beta 1\gamma 1$  were identified as integrin ligands, which are both present in the muscle junction, genetic data indicate that they play a relatively minor role at the muscle attachment site *in vivo*. The nature of the alternative  $\alpha$ PS1 $\beta$ PS and  $\alpha$ PS2 $\beta$ PS ligands has also remained elusive, and new, unexpected findings have emerged only recently. Firstly, it turns out that a protein synthesized in the tendon cells, *Drosophila* thrombospondin, depends on  $\alpha$ PS2 for its organization into the muscle matrix, and is thus one major  $\alpha$ PS2 $\beta$ PS ligand *in vivo* [90,91]. Secondly, the transmembrane muscle cell protein *perdido*, which curiously enough contains a laminin-like motif, appears to act as a ligand for  $\alpha$ PS1 $\beta$ PS on tendon cells [92]. To add further complexity to this picture, *perdido* interacts with the intracellular proteins Grip, which in turn might allow for *cis* interactions with the muscle integrin  $\alpha$ PS2 $\beta$ PS in the muscle plasma



**Fig. 3.** Schematic illustration of integrin–ligand interactions at the muscle–tendon cell junction in *Drosophila*. Binding of *perdido* to the integrin established a cell–cell contact. It is unclear how the ECM network is linked to the tendon side of this junction. dTSP; *Drosophila* thrombospondin.

membrane. These data thus show that the muscle–tendon cell linkage in *Drosophila* muscle at the molecular level is more intimate than was previously thought and involves an unexpected direct cell–cell mode of interaction (Fig. 3). The relationship between the muscle cell ligands laminin  $\alpha 2\beta 1\gamma 1$  and thrombospondin is currently unclear, however. Why do muscle integrins need two high-affinity ligands? It is possible that they could have different functions at different stages in muscle formation. The finding that some laminin mutations seem preferentially to affect the attachment of certain muscles [89] indicates that the relative contribution of different attachment mechanisms varies between muscle groups. Recently, a similar heterogeneity in laminin isoform expression has been shown in mouse extraocular muscle [93].

One lesson from the *Drosophila* system is thus that integrins in skeletal muscle serve as an important mechanical linkage at muscle cell junctions linking muscle cells to the basement membrane. Furthermore, *in vitro* studies of cultured embryonic *Drosophila* muscle cells demonstrated a role for integrins in myoblast fusion and sarcomere stability [84,94]. The molecular basis for these integrin-dependent effects on the cytoskeleton and differentiation is still not understood. The findings that soluble integrin ligands promote sarcomere formation might reflect activation of integrin signalling, indirectly affecting sarcomere formation. Whereas the integrin ligands in the *Drosophila* muscle junctions have only recently been clarified, the available knowledge about the ligands in mouse muscle is in some aspects more rudimentary (see below).

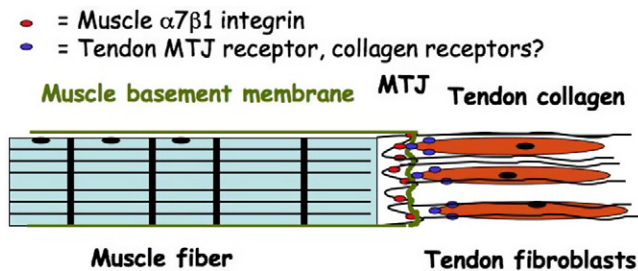
### 3.3. *M. musculus*

All the integrin genes have now been inactivated in mice [114–116], but our understanding of integrin function in mouse development is far from complete. In the case of embryonic or postnatal lethal phenotypes, the death of the animals precludes any detailed analysis of integrin function. We can expect to learn more about integrin function from conditional knockout animals in the next 10 years.

Some data have already started to emerge from detailed conditional knockout studies, however [117], although it should be remembered that knockout mice are not always the ideal animals when creating disease models and attempting close comparisons with the corresponding human condition. One striking example of this concerns dystrophin mutations, which are well tolerated in mice, whereas they cause a fatal condition in humans [118]. Another example illustrating the complexity of animal models is the recent

**Table 2**  
*Drosophila* integrin chains involved in muscle formation.

<i>Drosophila</i> integrin chain	Mutant	Phenotype	Extracellular ligands	Vertebrate orthologue(s)
$\beta$ PS3	<i>mys</i>	Embryonic lethal, muscle detachment	n.a.	$\beta 1$
$\alpha$ PS1	<i>multiple edematous wings (mew)</i>	Larval lethal, midgut defect	<i>Perdido</i> , laminin $\alpha 5\beta 1\gamma 1$	$\alpha 3$ , $\alpha 6$ , $\alpha 7$
$\alpha$ PS2	<i>inflated (if)</i>	Embryonic lethal, muscle detachment	Thrombospondin, tigrin, laminin $\alpha 2\beta 1\gamma 1$	$\alpha 5$ , $\alpha v$ , $\alpha 8$



**Fig. 4.** Schematic illustration of the myotendinous junction in the mouse. Muscle fibres are attached to the terminal basement membrane at the myotendinous junction (MTJ) via  $\alpha 7 \beta 1$  integrin. It is unclear how the fibroblasts are attached at the myotendinous junction.

work on cherubism in mice. A knockout model showed no phenotype in the bone of mice deficient in the adaptor protein SH3BP2, but transgenic animals with a disease mutation in the gene encoding this protein yielded a dramatic phenotype [119,120]. Great care must thus be taken when using a model organism to create disease models.

Based on the early findings in *Drosophila*, it was postulated that integrins would have similar functions in vertebrate muscle cells, i.e. affecting myoblast fusion and sarcomere stability [84]. Early experiments utilizing  $\beta 1$  integrin knockout cells isolated from chimeric mice nevertheless failed to show the myoblast fusion defect or sarcomere assembly defect in cultured cells [121]. Later work using a conditional knockout of  $\beta 1$  integrins in skeletal muscle did show that integrins are needed for myoblast fusion and sarcomere stability [122]. To explain this discrepancy it has been suggested that compensation mechanisms mask the defect during *in vitro* cell culture but that it is manifested *in vivo*. Likely candidates that probably cooperate in the muscle cells at various stages of myogenesis are the  $\alpha 4$ ,  $\alpha 5$  and  $\alpha 7$  integrin chains [123]. It is clear that some aspects of integrin function during the complex process of vertebrate myogenesis remain to be clarified.

$\alpha 5 \beta 1$  integrin [124] is a major embryonic/foetal muscle integrin during myogenesis *in vivo*, whereas  $\alpha 7 \beta 1$  is the important adult muscle integrin [125]. In the adult skeletal muscle sarcolemma the  $\alpha 7 \beta 1$  and dystroglycan receptors interact with different laminin isoforms.

In mouse, the number of laminin isoforms and the number of integrins have thus increased compared to the simpler fruit fly. During mouse myogenesis there are switches in the integrin and laminin isoforms being expressed, reflecting the need to control migration and cell adhesive strength. Except for a greater diversity to allow for different adhesion strengths, a new cell type has invaded the muscle in chordates/vertebrates, the fibroblast. In order to be able to interact with fibrillar collagens the fibroblast integrins have acquired an  $\alpha 1$  domain in their integrin  $\alpha$ -chains. In normal muscle the fibroblasts most likely take part in conditioning the muscle environment, controlling muscle gene expression and appearing as a passive player. In pathological situations the importance of these collagen-binding integrins in controlling the fibrotic response remains to be defined, but it is predicted to be substantial.

In the muscle sarcolemma integrins fulfil their functions at the junctions, whereas dystroglycan forms lateral contacts. Gene therapy approaches aimed at strengthening the muscle-basement membrane contact have been predicted to entail difficulties, mainly due to the delicate balance that exists between the laminin receptors in the lateral sarcolemma and in the junctions. Cell adhesion therapy in muscle is a challenging task that will require careful planning, including consideration of the effects of the various spliced forms of  $\alpha 7$  and  $\beta 1$  integrins in this scenario [126].

One aspect of muscle formation in vertebrates of which very little is known is muscle attachment site (MAS) formation at the tendon interface. In muscle, as in many other tissues, fibroblasts have received

little attention when it comes to tissue function. It has been recognized in stem cell biology that some stem cells use bi-directional signalling to establish a fibroblast stem cell niche [127] in order to maintain stem cell multipotency, and similar bi-directional signalling between cell types might also extend to muscle–tendon attachment sites. In skeletal muscle the muscle fibres make contact with the tendons at the myotendinous junctions (MTJ), but surprisingly little is known about how these contacts are made at the tendon side. Recent work with the transcription factor scleraxis that is expressed in the tendon/ligament has shed some light on tendon formation, but has also highlighted its complexity [128,129]. Although tendon and muscle can form independently of each other (to different degrees in muscles/tendons of different types), the two cell types are clearly dependent on each other for normal development. By analogy with the situation in *Drosophila*, it is possible that the muscle–tendon contact may depend on bi-directional signalling and may even involve direct tendon cell–muscle cell interactions at some stage. It will be important to define the molecules that are concentrated in the junctional fibroblasts at MTJs (Fig. 4), and it will also be interesting to identify the molecules involved in attaching the tendon to the bone. Future experiments will be certain to address this important issue, and the zebrafish might be a good model for studying tendon–bone attachments.

### 3.4. Outlook for other animal models in the study of integrin function in muscle

In addition to fruit fly and mouse, a number of additional animal models have the potential to be useful in elucidating integrin function during different aspects of myogenesis. Frogs have been extensively used to study the early steps in embryogenesis, including gastrulation [95–98]. Elegant work has led to the identification of fibronectin and of fibronectin-binding integrins as playing a role in organizing fibronectin during gastrulation [96]. A limited number of studies have been performed on integrins and muscle formation in *Xenopus*. One recent study showed that in *Xenopus* integrin-dependent somite formation depends on the proteins FAK and VASP [99]. If this is an evolutionary conserved function of integrins it is difficult to say at present. In mouse,  $\beta 1$  integrin<sup>−/−</sup> embryos die prior to somitogenesis [100], and the lack of somites in  $\alpha 5$ <sup>−/−</sup> embryos is secondary to mesodermal defects [101]. To our knowledge, no specific integrin mutation affecting somitogenesis specifically is known in mouse. Further studies of early steps of muscle formation might be informative in *Xenopus* (Table 3).

Another model organism that has seen a big increase in interest during the post-genomic era is the zebrafish (*D. rerio*) [102,103], the embryos of which are transparent, allowing easy visualization, and a number of mutants are available. The one complication that arises in genetic studies is related to ancient genome duplication, causing the

**Table 3**  
Evolutionary conserved functions of integrins in skeletal muscle.

Model organism	Integrin function in skeletal muscle	Evolutionary conserved	Usefulness of model
<i>Drosophila</i>	Cell adhesion (mechanical link) Sarcomere structure Myogenic differentiation	Yes	Studies of molecular mechanisms.
<i>Xenopus</i>	nd	nd	Early muscle development.
Zebrafish	Cell adhesion (somite border integrity)	?	Studies of muscle tendon attachments. Fin and muscle regeneration.
Mouse	Cell adhesion (Mechanical link) Sarcomere structure Myogenic differentiation	Yes	Muscular dystrophy models.

nd; not determined.



existence of multiple copies of some genes, which in many cases then seem to divide the functions between them. This always has to be borne in mind when trying to understand gene function in the zebrafish. Data on integrins in zebrafish are relatively scarce (50 references in Pubmed Oct. 2008), but one integrin that has been studied in some detail is the integrin  $\alpha 5$  orthologue (see below) [104,105]. The promising nature of this organism is illustrated in a recent paper in which a mutation screening focused on heart function identified laminin  $\alpha 4$  and ILK as candidate disease genes, a situation which was later validated in human patients [106]. Regarding skeletal muscle formation in zebrafish some studies have started to emerge on proteins previously implicated in human muscle disease [107,108]. Although some orthologues of muscular dystrophy genes are duplicated in the zebrafish, recent data on a dystrophin mutant fish (*sapje*) and a laminin  $\alpha 2$  mutant fish (*candyfloss*) have offered new insight into the early steps of muscle disease [109]. In the laminin  $\alpha 2$  mutation *candyfloss*, muscle fibre detachment with retained sarcolemma integrity was observed, suggesting that laminin  $\alpha 2$  is important for extracellular matrix integrity and that the detachment is an early step of the pathological process before the muscle fibres start to disintegrate. No information on zebrafish integrin  $\alpha 7$  mutations is available yet and the data on integrins in muscle in zebrafish is thus limited.

Integrin  $\alpha 5\beta 1$  mutations affect early muscle development and closer analysis suggest that this integrin is needed to maintain somite borders during the early steps of muscle formation [104]. Members of the TGF- $\beta$  family (Activin  $\beta A$ ) have been shown to be active during fin regeneration [110], and should offer a good system for studying integrin involvement in muscle regeneration. Finally, several mutations exist in the zebrafish mesoderm, which also affect the tendons, and some of these mutants might be interesting for studying the possible role of integrins in muscle–tendon attachments [111–113].

#### 4. Summary

In summary, we have shown that integrins have diversified and subspecialized during evolution. Current sequence data show that the origin of human-type integrin  $\alpha$  and  $\beta$  subunits arose after the divergence of the tunicates but orthologues, including multiple isoforms, are present in fish species. Analysis of sequence data from the genomes of shark species, the Florida lancelet and the sea lamprey may in the very near future clarify the likely point in chordate evolution when human-type homologues first arose. Key domains comprising the ectodomain of the  $\alpha$  and  $\beta$  subunits have early origins, with examples found in bacteria, whereas the origin and function of intact  $\alpha$  and  $\beta$  integrin subunits extend at least to the very earliest animals such as sponges. The I domain as an additional domain in some integrin  $\alpha$  subunits is a relatively late addition, found in the urochordates, whereas they are not found in echinoderms. Integrins with the I domain in tunicates are not orthologous to the vertebrate integrins having the I domain; in the later, there has been a clear differentiation into two groups, one set recognizing cells of the immune system (e.g.,  $\alpha L\beta 2$ ) and another that functions as collagen receptors (e.g.  $\alpha 2\beta 1$ ).

At least one  $\alpha$  subunit and one  $\beta$  subunit exist in sponges (e.g. *G. cydonium*), which lack tissues, muscle and nerves, but do have an embryonic state; already in cnidarians a network of nerves appear and both striated and smooth muscles have been observed. Characteristic of the diverging metazoans is this increase in complexity that probably has required additional integrins. As reflected in *Drosophila* with 5  $\alpha$  subunits and 2  $\beta$  subunits and human with 18  $\alpha$  subunits and 8  $\beta$  subunits, the integrin sequences, structures and their cellular functions have clearly evolved as metazoan organisms gained increasingly complex nervous systems, musculature, tissues and organs, a circulatory system and development of the adaptive immune system to complement the innate immunity present in invertebrates.

Thus, the increasing complexity of the metazoans appears to have gone hand-in-hand with an increase in the number of integrin subunits and the heterodimers that can be formed providing a means for their functional diversification.

Skeletal muscle is one of the oldest features of the metazoans as motility is a key feature of all animals at least during some stage of development. In *Drosophila*, the embryonic muscles are first tested for functionality when the embryos flex the muscles midway through embryogenesis, and integrins are necessary to form contacts between muscle cells and the cuticular exoskeleton. In vertebrates, muscle is integrated with a skeletal system where interstitial collagens appear as major proteinaceous building blocks of tendons, cartilage and bone. In order to be able to interact with these components, fibroblasts, chondrocytes and osteoblasts use integrins that, in contrast to *Drosophila* integrins, have picked up an  $\alpha I$  domain in order to anchor cells into this collagenous matrix [38,39]. During inflammation or muscle damage, infiltrating leukocytes and macrophages use  $\alpha I$  domain-containing  $\beta 2$  integrins to move into the muscle tissue.

The integrin linkage provides an attachment mechanism at muscle endpoints both in fruit flies and vertebrates. Surprisingly, in vertebrates some aspects of this linkage still remain unclear. Whereas the integrin  $\alpha PS2\beta PS$  or  $\alpha 7\beta 1$  integrins forms the muscle integrin linkage to laminin-containing basement membranes in adult invertebrate and vertebrate muscles respectively, virtually nothing is known about the linkage from the vertebrate tendon fibroblasts to the muscle attachment points. The future will tell if the vertebrate muscle cell-endoskeleton linkage shows conserved features at the molecular level with the invertebrate muscle cell-exoskeleton linkage and also involves integrins. It will also be interesting to see if the similarities to *Drosophila* muscle junctions extend to the perlecan-mediated mechanisms. Zebrafish might in the future be a useful model since several mesoderm mutants exist that might shed light on the molecular mechanisms involved in muscle–tendon linkages. Regarding the usefulness of the discussed animal models in helping to elucidate muscle disease mechanisms, zebrafish might also prove to be useful and in a not too distant future zebrafish might give useful clues to potential molecular targets to be stimulated in therapeutic approaches to muscle regeneration.

We have discussed in this review the molecular principles that have evolved within the integrin protein family and the molecular strategies that have been retained. As in all families, the processes of exploring the past and keeping the family heritage alive will continue to shed light on why the current family members are as they are.

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